Cell Density Plays a Critical Role in Ex Vivo Expansion of T Cells for Adoptive Immunotherapy

Hindawi Publishing Corporation
Journal of Biomedicine and Biotechnology
Volume 2010, Article ID 386545, 13 pages
doi:10.1155/2010/386545

Research Article

Cell Density Plays a Critical Role in Ex Vivo Expansion of T Cells for Adoptive Immunotherapy

Qiangzhong Ma,[1,2] Yawen Wang,[2] Agnes Shuk-Yee Lo,[2] Erica M. Gomes,[1] and Richard P. Junghans[1,2]

[1] *Biotherapeutics Development Laboratory, Roger Williams Medical Center, Boston University School of Medicine, Providence, RI 02908, USA*
[2] *Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA 02115, USA*

Correspondence should be addressed to Qiangzhong Ma, qma@rwmc.org

Received 24 December 2009; Revised 5 April 2010; Accepted 6 May 2010

Academic Editor: Zhengguo Xiao

The successful ex vivo expansion of a large numbers of T cells is a prerequisite for adoptive immunotherapy. In this study, we found that cell density had important effects on the process of expansion of T cells in vitro. Resting T cells were activated to expand at high cell density but failed to be activated at low cell density. Activated T cells (ATCs) expanded rapidly at high cell density but underwent apoptosis at low cell density. Our studies indicated that low-cell-density related ATC death is mediated by oxidative stress. Antioxidants N-acetylcysteine, catalase, and albumin suppressed elevated reactive oxygen species (ROS) levels in low-density cultures and protected ATCs from apoptosis. The viability of ATCs at low density was preserved by conditioned medium from high-density cultures of ATCs in which the autocrine survival factor was identified as catalase. We also found that costimulatory signal CD28 increases T cell activation at lower cell density, paralleled by an increase in catalase secretion. Our findings highlight the importance of cell density in T cell activation, proliferation, survival and apoptosis and support the importance of maintaining T cells at high density for their successful expansion in vitro.

1. Introduction

T cells are a critical component of the cellular immune response. In the past two decades, adoptive transfer of tumor reactive T cells into cancer patients has been created as an immunotherapy method to combat cancer [1]. This includes the early studies with lymphokine-activated killer (LAK) cells derived from ex vivo amplification of autologous lymphocytes with interleukin-2 (IL-2), late studies with tumor infiltrating lymphocytes (TILs) isolated from tumor specimens, and recent studies with genetically modified tumor reactive T cells [2]. The prerequisite for the success of adoptive immunotherapy relies on the successful ex vivo expansion of a large amount of T cells (up to 10^{11}).

The ex vivo expansion of T cells for adoptive immunotherapy usually involves two phases. The first phase is T cell activation, in which resting T cells are activated with anti-CD3 antibody or plus anti-CD28 antibody supplemented with IL-2. The second phase is activated T cell (ATC) proliferation. After activation, resting T cells become ATCs and undergo vigorous cell proliferation for about two to three weeks and the ATCs lose their proliferation capacity in about four weeks. Maintaining high cell density has been considered important among investigators performing ex vivo T cell expansions for clinical therapeutic applications. This report addresses formally the basis for this observation.

Cell density has been reported to be an important factor in maintaining certain T and B cells in vitro. Resting T cells die rapidly by apoptosis when cultured under diluted conditions but survive for extended periods when cultured at high cell density [3]. This effect was found to be mediated by soluble factors and independent of integrin-mediated signals. An acute T-lymphocytic leukemia cell line, CCRF-CEM, was reported to display a cell density-dependent growth characteristic [4]. CEM cells grow well at cell density $>2 \times 10^5$ cells per mL, but at low cell densities the

cultures rapidly undergo apoptosis. The viability of low-density CEM cells could be preserved by supplementing with "conditioned" medium from high-density CEM cultures. Catalase was identified as the active component in the conditioned medium. B cell chronic lymphocytic leukemia (CLL) was reported to be dependent on cell density for surviving in cultures [5]. CLL cells survival was strongly enhanced at high cell density. Conditioned medium from high cell density CLL cells produced a marked increase in the viability of low cell density autologous cells. Again, autocrine catalase was identified as the survival factor in the high cell density cultures.

Reactive oxygen species (ROS) have been shown to contribute to the death of CEM cells and CLL cells at low cell density [4, 5]. ROS are highly reactive metabolites that are generated during normal cell metabolism. Intracellular ROS derive mainly from leakage of electrons from mitochondrial electron transport chains that reduce molecular oxygen to superoxide ions. Cells possess antioxidant systems to control their redox state, to reduce oxidative stress and to maintain cell survival [6]. Superoxide ions are converted to hydrogen peroxide (H_2O_2) by the action of Cu^{2+}/Zn^{2+}-dependent or Mn^{2+}-dependent superoxide dismutases, and H_2O_2 is then detoxified by catalase or glutathione peroxidase. H_2O_2 can also react in vivo to generate the highly damaging hydroxyl radical by the Fe^{2+}-dependent Fenton reaction or the Fe^{2+}-catalyzed Haber-Weiss reaction [6, 7]. At subtoxic levels, ROS may play an essential signaling role in cell growth and differentiation [8–11]. At elevated levels, however, intracellular ROS are sufficient to trigger cell death [12–16]. Antioxidants that limit ROS-induced cell damage can suppress apoptosis in many systems. For example, N-acetylcysteine (NAC), which elevates intracellular glutathione levels, delays activation-induced cell death of a T cell hybridoma [17]. NAC or the iron chelator pyrrolidine dithiocarbamate (PDTC) or enforced expression of Mn^{2+}-dependent superoxide dismutase inhibits apoptosis induced by TNF-α which can stimulate ROS production [18–21]. Similarly, cell death through oxidative mechanisms has been shown to be opposed by protein albumin at physiologic concentrations directly by scavenging for free oxygen radicals through the free cysteinyl sulfhydryl moiety and indirectly by maintaining the reduced state of cellular proteins [22–24].

ROS have also been shown to be the decisive contributors to the death of activated T cells (ATCs) [25–28]. First, the ATCs have increased levels of ROS [25, 26, 29–31]. Second, ATC death is prevented by manganese (III) tetrakis (5, 10, 15, 20-benzoic acid) porphyrin (MnTBAP), an antioxidant that has been shown to inhibit ROS-induced death in different types of cells [25]. Evidence shows that ROS lead to ATC death by at least two pathways, one mediated by caspase activation and subsequent proteolytic cellular disintegration and the other driven by ROS themselves [25].

While cell density has been found to be important for the survival of resting T cell and certain but not all leukemic T and B lines, it remains unclear if normal T cells behave in a cell density-dependent manner during T cell expansion. In this study, we confirm a critical role of cell density in resting T cell activation and ATC expansion. We found that resting T cells need to be kept at high cell density for optimized activation and ATCs need to be kept at high cell density for optimized expansion. We show that the cell density-related ATC apoptosis is triggered by oxidative stress that is in turn opposed by the secretion of an autocrine survival factor, mainly catalase: at high cell density, more catalase accumulates in the medium and opposes the oxidative apoptosis. Further, we confirm the antioxidant activity of added NAC or of serum albumin at high concentrations that protects ATCs from apoptosis when cultured at low cell density.

2. Materials and Methods

2.1. Reagents. N-acetylcysteine (NAC), catalase, and 3′-amino-1,2,4-triazole (ATZ) were purchased from Sigma Chemical (St Louis, MO, USA). Human serum albumin was from Bayer Corporation (Elkhart, IN, USA).

2.2. Cell Purification, Activation, and Culture. Peripheral blood mononuclear cells (PBMCs) were isolated from venous blood from healthy adults by centrifugation over Histopaque-1083 (Sigma). For resting T cells activation study, PBMCs were cultured in 6-well plates in different cell densities and were activated in serum-free AIM V media (Gibco, Gaithersburg, MD, USA) supplemented with 100 U/ml IL-2, 100 ng/mL mouse antihuman CD3 antibody OKT3 (Ortho Biotech, Raritan, NJ, USA) only or with the addition of 100 ng/mL mouse antihuman CD28 antibody mAb9.3 (gift from Dr. Carl H. June, University of Pennsylvania, Philadelphia, PA, USA). For preparation of ATCs, PBMCs were activated at 1×10^6/mL cells in serum-free AIM V media supplemented with 100 U/ml IL-2, 100 ng/mL OKT3 in 75 cm^2 flasks for 3 days. ATCs were then washed and cultured in the AIM V medium supplemented with 100 U/mL IL-2 for an additional 7 days before use in experiments.

2.3. Monitor of T Cell Activation and Cell Division. Accompanied with T cell activation, there is a significant cell size enlargement from resting T cells to ATCs, with cytokines such as IL-2 and IFN-γ production and surface molecules such as CD69 and CD25 expression. ATCs can be distinguished from resting T cells and other types of PBMCs as the enlargement in forward-angle light scatter (FS) by flow cytometric analysis and it is identical with CD69 or CD25 staining. T cell activation was monitored by counting the percent of ATCs in the culture by flow cytometer and confirmed with microscope. To monitor cell division, ATCs were labeled with 1 μM carboxy-fluorescein diacetate succinimidyl diester (CFSE) (Molecular Probes, Eugene, OR, USA) [32] and cultured for 6 days. Cells were harvested and cell division was analyzed by the dilution of CFSE in the daughter cells by flow cytometer.

2.4. Detection of Apoptosis. ATC apoptosis was determined by cell shrinkage and DNA cleavage. Cell viability of ATCs was analyzed by flow cytometry [5, 33, 34]. Cell shrinkage

accompanying ATC apoptosis was detected as a reduction in forward-angle light scatter (FS) by flow cytometric analysis. Agarose gel electrophoresis was used to detect internucleosomal cleavage fragments of DNA following apoptosis [4, 35]. 2×10^6 cells were pelleted and resuspended in 500 μL of ice-cold lysis buffer (20 mM Tris·HCl, 10 mM EDTA, and 0.2% Triton X-100, pH 7.4). Proteinase K was added at 100 μg/mL and incubated at 50°C overnight, followed by further incubation at 37°C for 2 hours with the addition of RNase. DNA was extracted twice with phenol/chloroform at 1 : 1 and precipitated with isopropanol. The DNA was electrophoresed through a 1% agarose gel and stained with ethidium bromide.

2.5. Measurement of Intracellular Oxidative Stress. Cells were gently resuspended in 10 μM dihydrorhodamine 123 (DHR) (Molecular Probes) and incubated for 30 minutes and then analyzed by flow cytometry [5, 7]. The level of intracellular ROS was inferred from the mean fluorescence intensity (MFI) of DHR-stained cells. Dead cells and debris were excluded from analysis by electronic gating of forward and side scatter measurements.

2.6. Generation of Conditioned Medium. Cells were cultured at 1×10^6/mL (ATCs) or confluent (MIP101 cells, a human colon carcinoma cell line) in 75 cm^2 flasks. After 2 days in culture, the conditioned medium (CM) was removed from the culture flasks and centrifuged at 1500 rpm for 10 minutes. The CM was then passed through a sterile 0.45 μm filter.

2.7. Detection of Catalase. Detection of catalase was performed by western blot and dot blot. For western blot, control medium, ATCs CM, and cell lysates from ATCs were run on 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and electrotransferred to nitrocellulose membranes. For dot blot, 100 μL culture medium from untreated, anti-CD3 activated and anti-CD3 plus anti-CD28 activated PBMCs were blotted on to nitrocellulose membrane by using a dot blot apparatus (Bio-Rad, Hercules, CA, USA). The membranes were blocked with 5% nonfat dry milk in Tris-buffered saline (20 mM Tris/500 mM NaCl, pH 7.5) for 1-2 hours. Membranes were washed with TTBS (0.05% Tween-20 in Tris-buffered saline), and mouse antihuman catalase mAb antibody (Sigma) was added at a dilution of 1 : 1000 for 1 hour, followed by incubation with HRP-conjugated goat antimouse IgG antibody at a dilution of 1 : 1000 for 1 hour, all in 1% non-fat dry milk in TTBS. The membrane was developed with enhanced chemiluminescence reagent (Amersham, England, UK) and exposed to X-ray film for 5–60 seconds.

2.8. Detection of Intracellular Albumin. Analysis of intracellular albumin was performed using immunofluorescence and flow cytometry. Cells were fixed and permeabilized with a Fix & Perm kit (Caltag Laboratories, Burlingame, CA, USA) and then stained with mouse antihuman albumin (Sigma), followed by staining with a secondary goat antimouse FITC-conjugated antibody (Caltag Laboratories).

3. Results

The objective of this study was to determine the role of cell density in ex vivo expansion of T cells. To mimic the actual situation in preparation for clinical applications, resting T cells were not further purified from the bulk PBMCs in the experiments. Typically, PBMC are composed of 50–70% T cells, and smaller numbers of B cells, NK cells, and monocytes.

3.1. Cell Density Determines the Fate of T Cells Activation. Efficient activation of resting T cells requires signal 1 (TCR, CD3) and signal 2 (CD28) costimulation [36]. Activation of T cells through soluble OKT3 (anti-CD3) antibody depends on crosslinking of the antibody through Fc receptor on monocytes present in PBMCs preparations that also provide costimulation through B7 to CD28 on the T cells [37]. Typically, PBMCs cell concentrations of at least $\mathbf{1 \times 10^6}$/mL are specified for this T cell activation. At lower cell densities, the opportunities for cell-cell contact are diminished and activation is incomplete or absent.

This is confirmed in our results. Following activation with OKT3 and IL-2 at 1×10^6 cells/mL for 6 days, 61% of cells in the culture showed a T blastic morphology by flow cytometry versus 23%, 9%, and 8% for the lower PBMCs densities of 1×10^5, 1×10^4 and 1×10^3/mL, respectively (Figure 1). At an intermediate T cell density of 1×10^5/mL, addition of an agonist anti-CD28 antibody that bypasses need for B7 on monocytes partially compensates for the reduced cell contacts, doubling the activated fraction from 23% to 47%. At the lowest cell densities, this maneuver was not effective and at the highest it was unnecessary, where the activated phenotype was maximal with OKT3 alone. At higher density, there are more chances of cell contacts and B7-CD28 interactions, and costimulation is maximal without added anti-CD28 antibody. Such maximally activated T cells (ATC) were applied throughout this study.

As analyzed by flow cytometry from one typical experiment, the PBMCs isolated from normal human blood were 57.2% CD3+, 38.8% CD4+, 21.6% CD8+, 0.37% CD4+CD8+, 39.2% CD4−CD8−, 49.8% CD28+, 53.6% CD2+, and 60.6% CD11a+. After 3 days activation with OKT3 and expansion for 7 days (total 10 days), the cell populations were 99.7% CD3+, 81.7% CD4+, 15.6% CD8+, 1.9% CD4+CD8+, 0.76% CD4−CD8−, 99.4% CD28+, 99.5% CD2+, and 100% CD11a+. Such 10-day cultures were used in the ATC studies that follow.

3.2. ATCs Proliferate at High Cell Density. To examine the relation between cell density and ATC proliferation, T cells were activated under high density conditions as in Section 3.1 and then reseeded at varying cell densities and monitored over time for viable cell numbers. There was a progressive increase in the number of viable cells when ATCs were cultured at 1×10^5/mL, but a progressive decline in viable cells when ATCs were cultured at 1×10^4/mL. After 6 days, the number of viable ATCs increased 340% when cultured at 1×10^5/mL but decreased by 75% when cultured at $1 \times \mathbf{10^4}$/mL

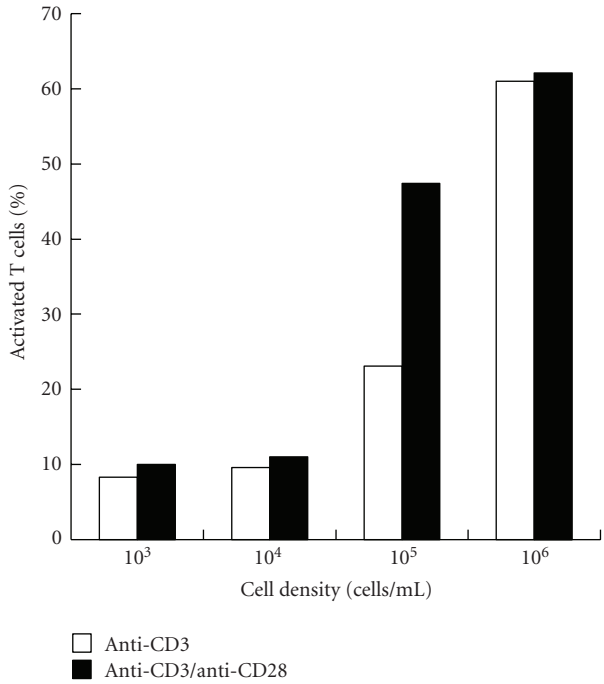

Figure 1: Cell density determines the fate of T cell activation. PBMCs cultured at different cell densities in 6-well plates were activated with anti-CD3 antibody alone or plus anti-CD28 antibody in the presence of IL-2. T cell activation was measured as percentage of activated T cells in culture after six days of activation by morphological criteria of T cell blasts on flow cytometer. Similar results were obtained in two independent experiments.

(Figure 2(a)). Furthermore, when measuring dividing cells by CFSE, 68% of ATCs underwent division when cultured at 1×10^5/mL, 35% at 1×10^4/mL, and only 15% at 1×10^3/mL (Figure 2(b)). These results establish a correlation between cell density and ATC expansion in vitro.

3.3. ATCs Undergo Apoptosis at Low Cell Density. To examine the relation between cell density and cell viability, ATCs cultured at different cell densities were evaluated after 24 hours. A condensed cell size by morphology on flow cytometry has been shown to correlate with apoptosis/cell death markers under experimental conditions close to our own [5, 33, 34] and was used to distinguish apoptotic from viable cells in our tests. At cell density of 1×10^5/mL, viable cells remained unchanged at 92% after 24 hours but declined to 62% and 34% when ATCs were cultured at 5×10^4/mL and 1×10^4/mL over the same time interval (Figure 3(a)). Increasing IL-2 100-fold from 100 IU/mL to 10,000 IU/mL did not rescue ATCs from death at low cell density (data not shown).

To determine by another measure whether ATCs died by apoptosis when cultured at low cell density, a DNA fragmentation assay was employed. Apoptosis is a distinctive form of cell death that occurs in a wide range of physiological and pathological situations [38]. It differs fundamentally from degenerative cell death or necrosis and was originally defined by the orderly sequence of ultrastructural changes that accompanies cell elimination during development [39]. Biochemically, it is best characterized by the presence of internucleosomal cleavage of DNA into 180–200 base-pair fragments [39, 40], which can be demonstrated by gel electrophoresis. As shown in Figure 3(b), DNA was fragmented when ATCs were cultured at low cell density (1×10^4/mL) but remained intact at high cell density (1×10^6/mL), confirming that apoptosis is the mechanism of ATC death at low cell density.

3.4. ROS Are the Mediators of ATC Apoptosis at Low Cell Density. Elevated intracellular ROS can trigger cell death [12–16]. We hypothesized that ROS were the mediators of ATC apoptosis at low cell density. To test this hypothesis, we measured the levels of intracellular ROS in ATCs cultured at different cell densities and tested if antioxidants can block ATC apoptosis at low cell density.

ROS include superoxide and hydroxyl-free radicals and H_2O_2. To measure intracellular ROS, we used the oxidation-sensitive fluorescent probe DHR [7]. DHR is nonfluorescent, uncharged, and accumulates within cells, whereas R123, the product of intracellular DHR oxidation, is fluorescent, positively charged, and trapped within cells [41]. ATCs cultured at high cell density (1×10^5/mL) and low cell density (1×10^4/mL) for 24 hours were incubated with DHR, and R123 fluorescence was measured by flow cytometry. The rate of DHR oxidation was significantly greater in ATCs cultured at low cell density than in ATCs cultured at high cell density (Figure 4(a)), confirming a correlation of intracellular ROS with apoptosis.

Antioxidants inhibit ROS-mediated apoptosis in many systems by limiting ROS-induced cell damage [16]. We therefore tested the effects of three antioxidants on ATC apoptosis at low cell density. NAC elevates intracellular glutathione [17] that is a substrate for glutathione peroxidase to catalyze the breakdown of H_2O_2; catalase detoxifies H_2O_2 produced by superoxide ions [7]; and albumin can directly scavenge reactive oxygen species through its free cysteinyl—SH [42]. ATCs were cultured at low cell density (1×10^4/mL) in the presence of antioxidants at different concentrations for 24 hours. Cell viability and intracellular ROS were measured by flow cytometry. All agents significantly protected cells from death in a dose-dependent manner. In general, the increased survival was paralleled by a reduction in intracellular ROS levels with higher levels of the antioxidants (Figure 4(b)). This further supports the hypothesis of ROS as a mediator of apoptosis in ATCs cultured at low cell density.

Albumin is the most abundant plasma protein and has been suggested to constitute an important extracellular antioxidant [43, 44]. Interestingly, the reduction in ROS with albumin appeared somewhat less than predicted versus the survival benefit observed (Figure 4(b)), suggesting that there may be additional, downstream means by which albumin may mitigate the harmful effects of ROS. A further feature of interest was the demonstration of increased albumin endocytosis in the low density ATCs versus those grown at high density (Figure 4(c)). Whether this was a generalized effect on endocytosis or selectively related to albumin was not

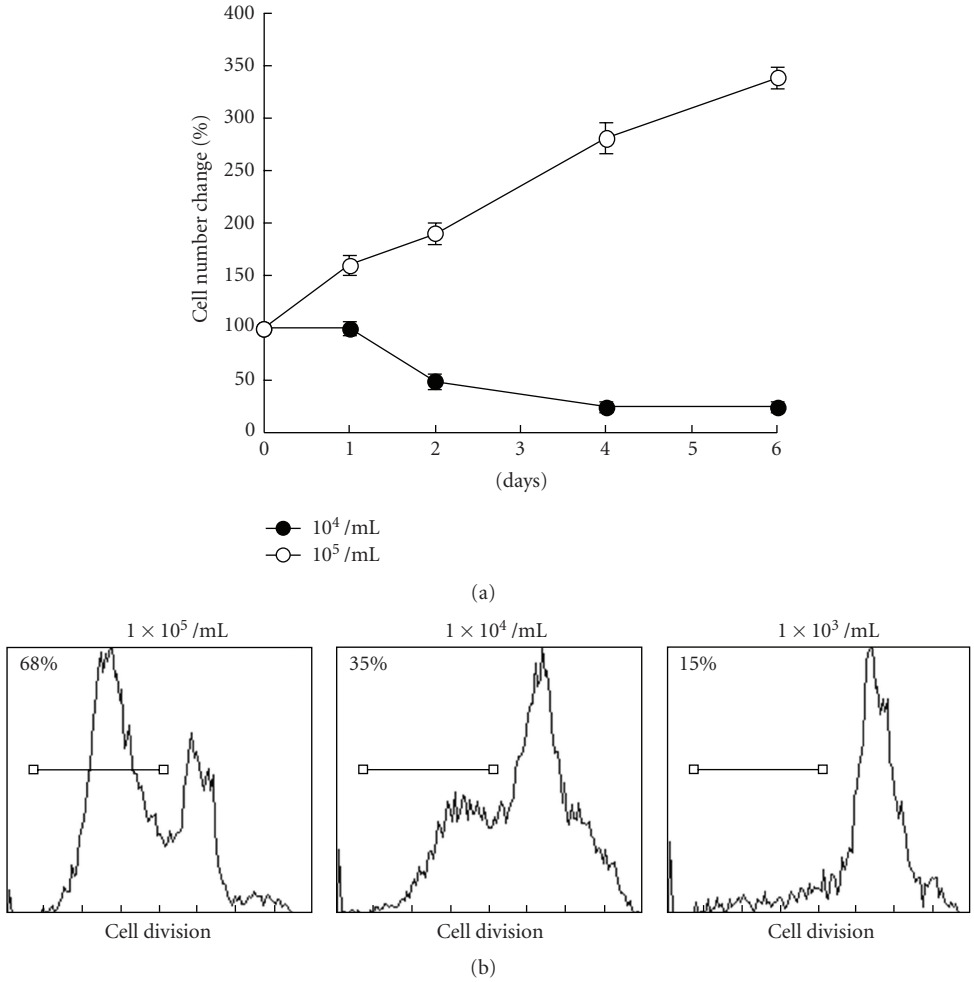

Figure 2: ATCs proliferate at high cell density. ATCs were activated at high density and then reseeded in fresh growth medium plus IL-2 at varying concentrations. (a) Numbers of ATCs at different cell densities. ATCs were cultured at high cell density (1×10^5/mL) or low cell density (1×10^4/mL) in 75 cm^2 flasks for 6 days. Cell number change rate (%) was monitored by direct cell counting. (b) Cell division of ATCs at different cell densities. After labeling with CFSE, ATCs were cultured at 1×10^5/mL, 1×10^4/mL, and 1×10^3/mL in 75 cm^2 flasks for 6 days. Cell division was monitored by CFSE dilution in the dividing cells as analyzed by flow cytometry.

examined. It was also not discriminated whether it was the internalized albumin or the external protein that mediated the survival benefits; its mechanism of action was not further investigated.

3.5. Soluble Factor Secreted by ATCs at High Cell Density Prevents Apoptosis of ATCs at Low Cell Density. The protective effect of high cell density indicated that either cell-to-cell contacts or soluble factor(s) produced by ATCs were inhibiting ATC apoptosis under this condition. To assess whether soluble factor(s) were involved, conditioned medium from ATCs cultured at high cell density (1×10^6/mL) was collected and added to ATCs cultured at low cell density (1×10^4/mL). With increased fractions of conditioned medium in the total medium, the intracellular ROS levels decreased in ATCs cultured at low cell density and their viability increased (Figure 5(a)). These data confirmed the secretion of one or more soluble factors at high cell density that functioned as antioxidant to protect ATCs from apoptosis.

A similar protective effect was observed with conditioned medium from a human colon carcinoma cell line, MIP101 (Figure 5(b)) and from a human T cell leukemia cell line (data not shown). These results indicate that the protective soluble factor(s) secreted by ATCs at high cell density are not ATC-specific. This result is compatible with the potential of diverse cell lines to function as feeder cells during T cell cloning procedures [40].

3.6. Autocrine Catalase Protects ATCs from Apoptosis. Based on prior studies of cytoprotective effects of catalase in cultures of lymphoid leukemia cells [5, 40], we hypothesized that the cell density effect on ATC survival was also mediated by secreted catalase. To determine whether catalase was one of the autocrine survival factors, western blot was performed (Figure 6(a)). The enzyme was clearly detected in cell lysates

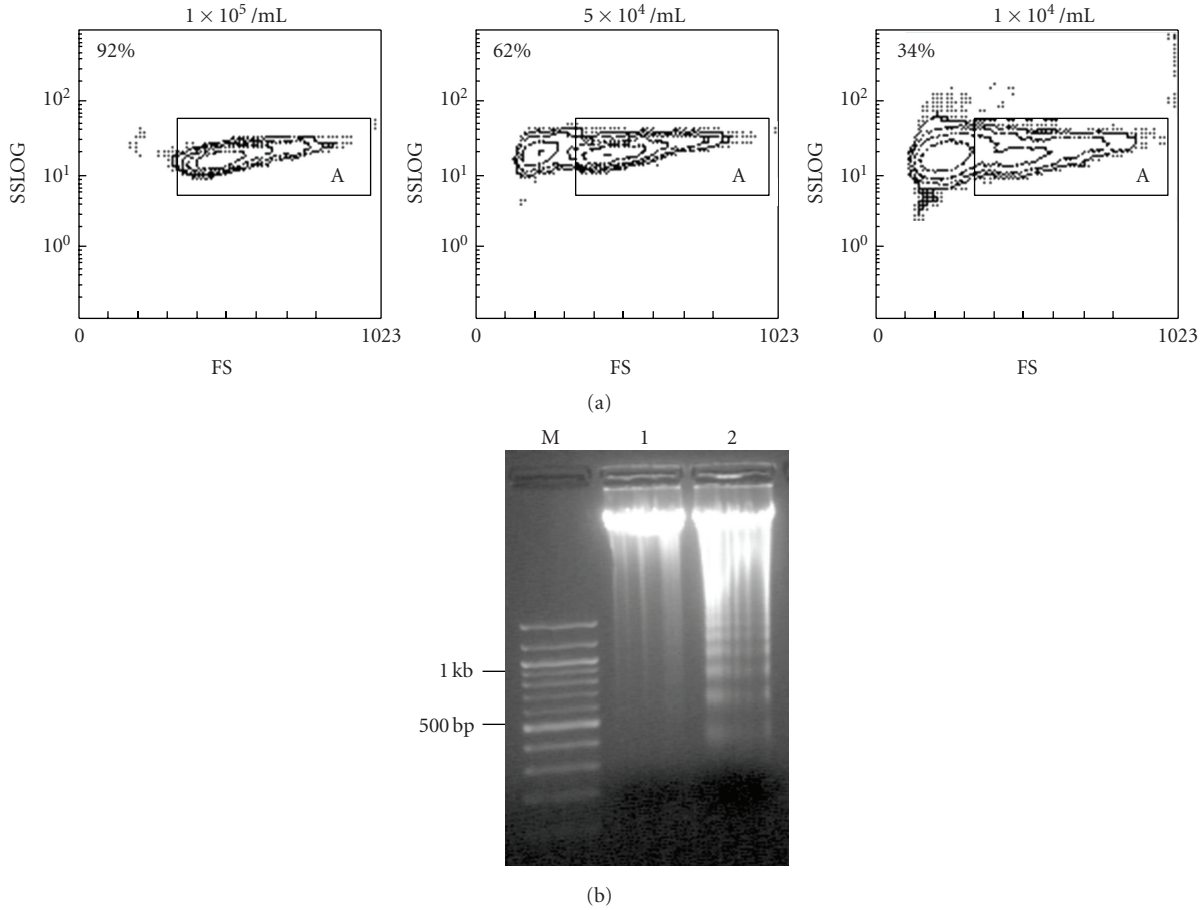

Figure 3: ATCs undergo apoptosis at low cell density. (a) Cell viability of ATCs at different cell densities. ATCs were cultured at different cell densities: 1×10^5, 5×10^4, and 1×10^4/mL in 75 cm^2 flasks for 24 hours. Cell viability was analyzed by flow cytometry. Viable cells are included in the rectangle and percentages of viable cells are indicated within the icon top left. Similar results were obtained in 3 independent experiments. (b) DNA fragmentation of ATCs at different cell densities. The DNA extracts of ATCs cultured at high (1×10^6/mL) (lane 1) or low (1×10^4/mL) (lane 2) cell density in 75 cm^2 flasks for 24 hours were electrophoresed through a 1% agarose gel and stained with ethidium bromide. 100 bp DNA ladder markers were included as markers (M).

and conditioned medium, but not in control medium. In two separate assays, catalase in CM (e.g., lane 2) was estimated at 2.5–5 μg/mL (6–12 units/mL) in comparison with control purified catalase. Estimates from cell lysate (lane 3) are 2 μg per 10^6 cells. This means that in 24 hours 10^6 cells secrete into 1 mL of medium, a quantity (2.5–5 μg) that equals or exceeds what is present in the cells themselves. Further, this concentration of catalase in CM corresponds closely with levels that give maximum benefit to ROS control and cell viability in Figure 4(b) (e.g., ~10 units/mL).

We next sought to establish whether catalase contributed to the survival-enhancing effect of the conditioned medium. To address this question, the effect of the selective catalase inhibitor, 3′-amino-1,2,4-triazole (ATZ) [5, 45–48] was examined. ATZ significantly blocked the survival-enhancing activity of the conditioned medium (Figure 6(b)), indicating that catalase plays the major role in this cytoprotective effect.

3.7. Elevated Autocrine Catalase Accumulation in CD28 Costimulated T Cell Activation. Having established the role of autocrine catalase as a cell survival factor in ATC proliferation, an interesting question arises: whether autocrine catalase also plays a role in CD28 costimulated T cell activation. An intermediate cell concentration was shown in Figure 1 to benefit from CD28 costimulation, PBMCs at 1×10^5/mL were activated with anti-CD3 antibody without or with anti-CD28 antibody in the presence of IL-2. Compared with anti-CD3 antibody activation alone, there was a significant more amount of autocrine catalase accumulation in the anti-CD28 antibody costimulated cell cultures (Figure 7). These results indicate that the improved T cell activation at lower cell density with CD28 is paralleled by an enhanced autocrine catalase secretion.

4. Discussion

Cell density has been reported to be important for cell survival in cultures of resting T cells [3] and certain leukemic T and B cell lines [4] but not reported in other leukemic T cell lines such as Jurkat and H9 T cells [5]. Although

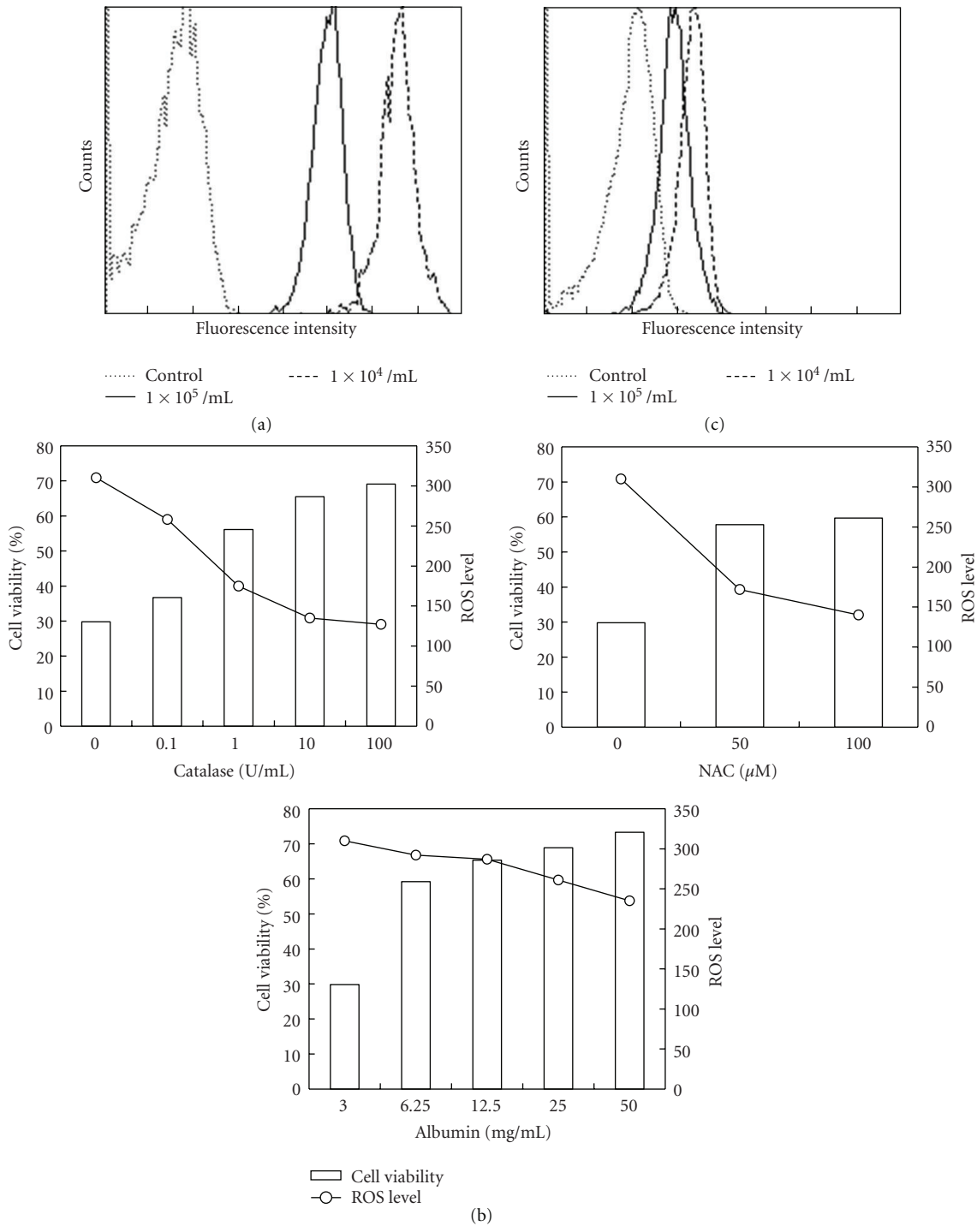

Figure 4: Reactive oxygen species (ROS) are the mediators of ATC apoptosis at low cell density. (a) Intracellular ROS levels in ATCs cultured at different cell densities. ATCs were cultured at high cell density (1×10^5/mL) or low cell density (1×10^4/mL) in 75 cm^2 flasks for 24 hours. After staining with DHR for 30 minutes, intracellular ROS levels of ATCs were analyzed by flow cytometry. Nonstaining ATCs were used as negative control. (b) Antioxidants protect activated T cells from apoptosis at low cell density. ATCs were cultured at 1×10^4/mL in 75 cm^2 flasks in the presence of catalase, NAC, and human serum albumin (HSA) in different concentrations. HSA in unsupplemented serum-free medium was measured at 3 mg/mL by Bradford assay. Both cell viability and intracellular ROS levels were analyzed by flow cytometry as in (a). ROS levels were indicated as mean fluorescence intensity (MFI) of DHR-stained cells. Similar results were obtained in 2 independent experiments. (c) Elevated intracellular albumin in ATCs cultured at low cell density. ATCs were cultured at high cell density (1×10^5/mL) or low cell density (1×10^4/mL) in 75 cm^2 flasks for 24 hours. Cells were fixed and permeabilized and then stained with mouse antihuman albumin antibody, followed by staining with a secondary goat antimouse FITC-conjugated antibody. Cells stained with only secondary antibodies were used as negative controls.

there are frequent communications between investigators performing ex vivo T cell expansions for clinical therapeutic applications that maintaining high cell density is an important consideration factor in T cell expansion, the relation between cell density and T cell expansion remains unclear. In this study, we systematically examined the relation between cell density and normal human T cell expansion in vitro, providing evidence for optimizing T cell expansion protocols for clinical applications. From our results, PBMCs have to be seeded at high cell density ($\geq 1 \times 10^6$/mL) for optimal T cell activation. The addition of CD28 costimulation helps resting T cells to be activated at lower cell density and coordinately yields elevated catalase secretion and accumulation in the cultures. Maintaining high cell density is also important for ATC proliferation. ATCs undergo apoptosis when cultured at cell density of 1×10^4/mL or less. Our mechanistic studies support the role of ROS and oxidative stress apoptosis in ATC death at low density. At high cell density, the extracellular accumulation of secreted catalase reduces intracellular ROS species and alleviates their toxic effects.

4.1. Cell Density Plays a Critical Role in Ex Vivo Expansion of T Cells. Current protocols for ex vivo expansion of T cells for clinical adoptive immunotherapy usually involve the activation of PBMCs with OKT3 alone or plus anti-CD28 antibody in the presence of IL-2. When expanding T cells in vitro, our results indicate that it is critical to maintain the cells at high cell density during both T cell activation and ATC expansion phases. It is reported that OKT3 has to be immobilized on plastic or crosslinked via accessory cells in PBMCs through Fc receptor binding for the activation of T cells [37]. At low cell density, where the cell-cell contact is poor, the OKT3 may not be efficiently crosslinked to activate resting T cells. We also found that the addition of costimulation through anti-CD28 antibody improves the activation of resting T cells at lower cell density. Unlike the OKT3, the soluble form of mAb9.3 can induce costimulation directly without the need for crosslinking [37]. Interestingly, there is a correlation between CD28 costimulation and elevated catalase accumulation in the cultures. At present, there is no basis on which to infer whether catalase is in the CD28 pathway or merely one of the many downstream components of T cell activation. Once being activated, the ATCs have to be kept at high cell density for survival and efficient proliferation.

When T cells are activated, T cells secrete abundant cytokines such as IL-2 and other growth factors for cell proliferation and survival. Maintaining high cell density in culture may enable the accumulation of such autocrine products to reach a relatively high concentration to support the proliferation and survival of ATCs. It is interesting to notice that under physiological conditions, T cell immune responses can only be exclusively induced in organized lymphoid tissues but not elsewhere in the body [49–53]. Naïve T cells traffic constitutively through secondary lymphoid organs where they encounter antigen-loaded dendritic cells and are activated to proliferate and differentiate into activated effector T cells. Following this, effector T cells then migrate to peripheral tissues to perform their function

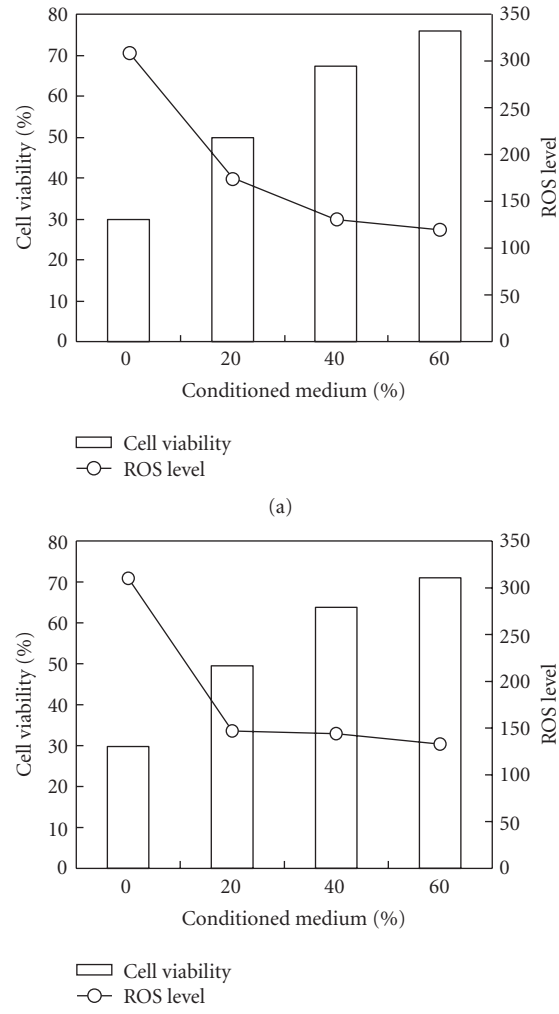

Figure 5: Soluble factors secreted by ATCs at high cell density prevent apoptosis of ATCs at low cell density. ATCs cultured at 1×10^4/mL in 75 cm^2 flasks were supplemented with conditioned medium from (a) ATCs at high cell density or (b) MIP101 cells in different concentrations for 24 hours. Cell viability and intracellular ROS levels were analyzed by flow cytometry. Similar results were obtained in two independent experiments.

[54]. Is it true that at locations such as the T cell areas of secondary lymphoid organs, high cell density enables the initiation of T cell responses with supported T cell survival and proliferation, whereas at sites of peripheral tissues, low cell density prevents T cell proliferation and minimizes immunopathology? The question remains to be answered.

4.2. ROS Are the Mediators of ATC Apoptosis at Low Cell Density. To understand why T cells have to be maintained at high cell density for optimal expansion, it is important to know why the T cells die at low cell density. ROS such as superoxide and hydroxyl radicals and H_2O_2 are continuously produced by cells, and their levels are regulated by a number of enzymes and physiological antioxidants. Excessive generation of ROS or failure to suppress elevated

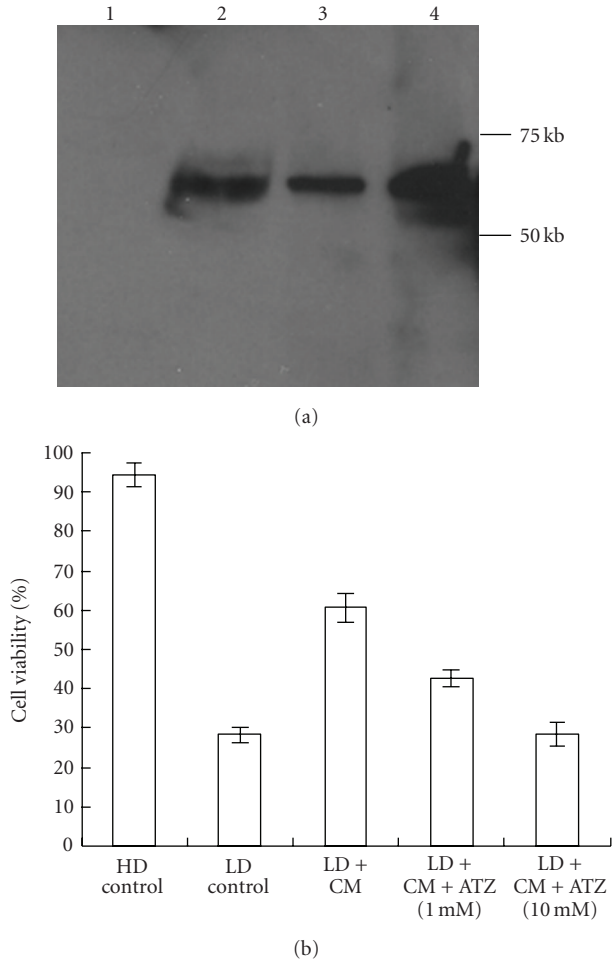

Figure 6: Autocrine catalase is the conditioned medium product that protects ATCs from apoptosis. (a) Expression and secretion of catalase by ATCs. Western blot of control medium (lane 1), conditioned medium from ATCs (lane 2), cell lysates of ATCs (lane 3), and catalase control (lane 4) probed with anticatalase antibody. (b) Catalase inhibitor ATZ abrogates the protective effect of conditioned medium from ATCs. ATCs were cultured at high cell density (1×10^5/mL) (HD) or low cell density (1×10^4/mL) (LD) in 75 cm^2 flasks in the presence or absence of 50% conditioned medium from ATCs (CM) and ATZ in 1 or 10 mM for 24 hours. Cell viability was analyzed by flow cytometry.

intracellular ROS by the cellular regulatory systems has been associated with cell death [12–15].

Differential effects of ROS on cell death are observed depending on the level of ROS within the cell [26, 55]. High levels of ROS lead to lipid peroxidation, damage to cellular membranes, inactivation of caspase enzymes, and necrotic cell death. Low levels of ROS can activate protein kinases and phosphatases, mobilize Ca^{2+} stores, activate or inactivate transcription factors, and lead to apoptotic cell death. ATCs have been shown to have increased levels of ROS [25, 26, 29–31] and ROS have been shown to be one of the decisive contributors to the death of ATCs [25–28].

ROS are intermediates in the induction of FasL after TCR engagement during activation induced cell death [25]. ROS-driven Bcl-2 downregulation is a necessary signal for activated T cell autonomous death [25]. Besides, ROS may affect many other molecules, such as membrane lipids, transcriptional factors, and signal transduction proteins that are involved in T cell apoptosis [25].

Our data demonstrate that ATCs cultured at low cell density have higher levels of ROS than ATCs cultured at high cell density and that reversal of high ROS in culture improves T cell proliferative response and survival, implying that ATC apoptosis at low cell density is triggered by ROS.

4.3. Antioxidants Promote ATC Survival at Low Cell Density. Glutathione (GSH) is the major intracellular redox buffer and plays an essential role in protecting cells against oxidative damage [56]. In addition, changes in the intracellular GSH levels modulate the expression of several genes involved in the control of cell growth and differentiation [57, 58]. In T lymphocytes, intracellular GSH is critical for the proliferative response to mitogens or antigens [59–62]. Our experiments demonstrate that by supplementing the GSH precursor, NAC, ATCs can be protected from apoptosis at low cell density, suggesting that the GSH peroxidase antioxidant system may play an important role in ATC survival.

Other interventions that reduce intracellular ROS were also effective in reversing the effect of low cell density to inhibit T cell proliferation and survival. These included the supplementation of cultures with purified catalase, which detoxifies H_2O_2, and addition of high concentrations of serum albumin, which contributes reducing cysteines that can scavenge oxygen radicals.

One of the striking features of human serum albumin is the presence of 34 cysteine residues forming 17 disulfide bonds, and one free thiol at the Cys-34 position [63]. One-third of the albumin molecules form mixed disulfides with either GSH or half-cystine. The remaining sulfhydryl group of the Cys-34 residue of albumin constitutes the major extracellular source of reactive free thiol [64]. In this context, it has been suggested that albumin constitutes an important extracellular antioxidant in plasma [43]. The role of albumin as an ROS scavenger has been confirmed in cell-free systems with a wide variety of oxidative species, including HOCl, H_2O_2, $^{\bullet}OH$, carbon radicals, and peroxynitrite [22, 65, 66], as well as in intact cell systems such as macrophages and renal tubular epithelium [42]. However, the mechanism by which albumin exerts its antioxidant effects is most likely multifactorial [42]. It is possible that the free sulfhydryl group of albumin enables it to act not only as an antioxidant but also as a reducing agent via modulation of cellular GSH levels [44]. GSH in turn affects a wide variety of cell proteins, the function of which is dependent on redox state, such as the N-methyl-D-aspartic acid receptor, the DNA binding protein activator protein-1, and NF-κB [67, 68].

Albumin has been an essential component of non-serum culture mediums for expansion of T cells for clinical applications. We explored the antioxidant role of albumin in cell density-related death of ATCs. Concentration-dependent reduction of intracellular ROS in ATCs cultured at low cell density suggests that albumin reacted as an antioxidant to

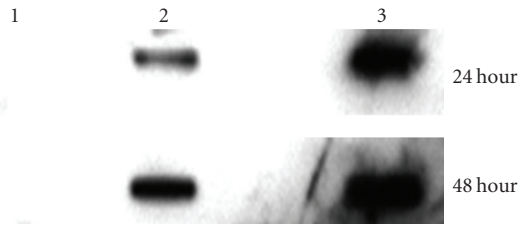

Figure 7: Improved activation with CD28 costimulation at low cell density is accompanied by higher autocrine catalase secretion. Slot blot of culture media from unactivated (lane 1), anti-CD3 antibody (lane 2) and anti-CD3 antibody plus anti-CD28 antibody (lane 3) activated PBMCs at 1×10^5/mL at 24 and 48 hours probed with anticatalase antibody.

scavenge ROS. Compared to antioxidants catalase and NAC, however, the effectiveness of albumin in reducing ROS is not as strong, compatible with its action via these other indirect mechanisms to rescue ATCs from apoptosis. The mechanism of albumin's action was not investigated further.

Interestingly, elevated intracellular albumin was detected in ATCs cultured at low cell density, implicating increased endocytosis under this condition, possibly in response to elevated intracellular ROS. Albumin is bound on the surface of lymphoid cells of all mammalian species tested [69], but its function is relatively unknown. ATCs express albumin binding proteins and the binding of serum albumin increased considerably upon blastic transformation [70]. Albumin is endocytosed and the internalized albumin is detected in peroxidase-conjugated form in lysosome-like bodies by ultrastructural cytochemistry. Pulse-chase experiments show that internalized albumin is finally released mainly in a degraded form from the cells [70]. Whether increased endocytosis of albumin under low-density conditions is an adaptive response to elevated intracellular ROS to import further reducing species into the cell is uncertain and was not investigated further.

It is likely that those nontoxic antioxidants such as sodium pyruvate [71], β-mercaptoethanol [72], DL-penicillamine and thiolactate that exhibit protective effects against ROS may have the same effect to protect ATCs from apoptosis at low cell density. Whether these agents are useful in the maintenance and growth of ATCs remains to be determined.

4.4. Autocrine Catalase Protects ATCs from Apoptosis. Among the naturally occurring variables examined, only one correlated with the reversal of high levels of ROS under conditions of high ATC cell density: the accumulation of secreted catalase that raised extracellular levels of this potent antioxidant enzyme. This extends studies of cultured leukemic T and B cells [4, 5] suggesting that autocrine catalase functions as a cytoprotective antioxidant in protecting cells at high cell density from apoptosis. Furthermore, our results and others' indicate that this factor is not cell-type restricted and is compatible with the observation that so-called "feeder" cells of various origins can function to support T cells under single-cell cloning conditions. We may infer that secretion of catalase to suppress intracellular oxidative stress is a key component of the supportive role of feeder cells.

How catalase is released from the cells is unclear. Catalase lacks a leader sequence and cannot therefore be secreted by the classic endoplasmic reticulum-Golgi secretory pathway [73]. It is suggested that, like some cytokines, catalase may be secreted via a leaderless secretory pathway [5]. It is unlikely that the appearance of catalase in the medium derives from dying cells. First, at high cell density, ATCs only begin to die at late stages of expansion (after 3-4 weeks). The CM we collected are from ATCs cultured at early stage of expansion (2 weeks) with good viability. Second, we have shown that CM from other cell lines that have very limited cell death can also prevent ATC apoptosis.

It was noted that the endogenous cellular catalase was similar in quantity to that secreted by the cells in 24 hours. Whether the intracellular enzyme is in a compartment that is functional or inactive is not addressed by these studies. However, it is clear that cellular catalase is not a substitute for the secreted component for maintaining cellular health; otherwise, the cell concentration effects would not be observed and CM would not rescue lower ATC densities from apoptosis.

Finally, the quantities of catalase in the CM (6–12 units/mL) (Figure 6(a)) corresponded closely to levels of purified catalase (~10 units/mL) that provide maximum control of ROS and high ATC viability (Figure 4(b)).

Regarding how extracellular catalase activity might regulate intracellular oxidative stress, it is suggested that the extracellular decomposition of H_2O_2 may create a concentration gradient favoring the diffusion of H_2O_2 out of the cells [5].

A final speculation is warranted on the relevance of these findings to the in vivo setting. The autocrine of catalase by T cells may be an important factor for their proliferation. At the time of activation, ATCs are at the highest level for ROS generation and most vulnerable to oxidative cell death [29]. At sites of high cellular density and low fluid efflux, such as lymph node paracortex, catalase could be expected to accumulate to high levels and support ATCs viability and proliferation. On the other hand, ATCs trafficking through the peripheral circulation will be dependent upon blood/tissue levels of ROS and tissue secretion of catalase, as well as serum albumin.

Another question remains to be answered is that the elevated ROS in ATCs and an increased rate of their death at low cell density may be a result of or partly related to excessive oxygen concentration in the growth medium cultivating in traditional incubators in comparison with oxygen concentration in the blood. Nevertheless, our studies clearly indicate that it is important to keep an antioxidant environment for optimized expansion of T cells in vitro.

In conclusion, the present study examined the role of cell density in T cell expansion in vitro. Several conclusions are drawn from this work. First, we confirm that cell density plays a critical role in T cell activation and ATC proliferation. Resting T cells were activated to expand at high cell density but failed to be activated at low cell density. ATCs grew

rapidly at high cell density but underwent apoptosis at low cell density in culture. Second, apoptosis of ATCs cultured at low cell density correlated with elevated intracellular ROS levels and was reversed by antioxidants NAC, catalase, and albumin, indicating that the apoptosis of ATCs at low cell density was mediated by ROS. Third, the increased survival of ATCs at high cell density was due to non-IL-2 factor(s) secreted by ATCs and non-ATCs alike. Fourth, autocrine catalase was demonstrated to be the key survival factor regulating ATC survival at high density by suppressing intracellular ROS. Fifth, CD28 costimulation that improves T cell activation at lower cell density is accompanied by enhanced autocrine catalase secretion. Our findings highlight the importance of cell density in T cell activation, proliferation, survival, and apoptosis and suggest that it is critical to maintain T cells at high cell density for the successful expansion of T cells ex vivo for adoptive immunotherapy.

Acknowledgment

This work was supported by Grant W81XWH-09-0039 from the Breast Cancer Research Program (BCRP) of the U.S. Department of Defense (DOD).

References

[1] S. A. Rosenberg, "Development of effective immunotherapy for the treatment of patients with cancer," *Journal of the American College of Surgeons*, vol. 198, no. 5, pp. 685–696, 2004.

[2] Q. Ma, R. M. Gonzalo-Daganzo, and R. P. Junghans, "Genetically engineered T cells as adoptive immunotherapy of cancer," *Cancer Chemotherapy and Biological Response Modifiers*, vol. 20, pp. 315–341, 2002.

[3] D. Pilling, A. N. Akbar, N. Shamsadeen, D. Scheel-Toellner, C. Buckley, and M. Salmon, "High cell density provides potent survival signals for resting T-cells," *Cellular and Molecular Biology*, vol. 46, no. 1, pp. 163–174, 2000.

[4] P. A. Sandstrom and T. M. Buttke, "Autocrine production of extracellular catalase prevents apoptosis of the human CEM T-cell line in serum-free medium," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 90, no. 10, pp. 4708–4712, 1993.

[5] E. C. Moran, A. S. Kamiguti, J. C. Cawley, and A. R. Pettitt, "Cytoprotective antioxidant activity of serum albumin and autocrine catalase in chronic lymphocytic leukaemia," *British Journal of Haematology*, vol. 116, no. 2, pp. 316–328, 2002.

[6] B. Halliwell and J. M. C. Gutteridge, "Role of free radicals and catalytic metal ions in human disease: an overview," *Methods in Enzymology*, vol. 186, pp. 1–85, 1990.

[7] G. Packham, R. A. Ashmun, and J. L. Cleveland, "Cytokines suppress apoptosis independent of increases in reactive oxygen levels," *Journal of Immunology*, vol. 156, no. 8, pp. 2792–2800, 1996.

[8] N. Peunova and G. Enikolopov, "Nitric oxide triggers a switch to growth arrest during differentiation of neuronal cells," *Nature*, vol. 375, no. 6526, pp. 68–73, 1995.

[9] K. Z. Guyton, Y. Liu, M. Gorospe, Q. Xu, and N. J. Holbrook, "Activation of mitogen-activated protein kinase by H_2O_2: role in cell survival following oxidant injury," *Journal of Biological Chemistry*, vol. 271, no. 8, pp. 4138–4142, 1996.

[10] E. M. Mills, K. Takeda, Z.-X. Yu, et al., "Nerve growth factor treatment prevents the increase in superoxide produced by epidermal growth factor in PC12 cells," *Journal of Biological Chemistry*, vol. 273, no. 35, pp. 22165–22168, 1998.

[11] K. Suzukawa, K. Miura, J. Mitsushita, et al., "Nerve growth factor-induced neuronal differentiation requires generation of Rac1-regulated reactive oxygen species," *Journal of Biological Chemistry*, vol. 275, no. 18, pp. 13175–13178, 2000.

[12] S. V. Lennon, S. J. Martin, and T. G. Cotter, "Dose-dependent induction of apoptosis in human tumour cell lines by widely diverging stimuli," *Cell Proliferation*, vol. 24, no. 2, pp. 203–214, 1991.

[13] D. M. Hockenbery, Z. N. Oltvai, X.-M. Yin, C. L. Milliman, and S. J. Korsmeyer, "Bcl-2 functions in an antioxidant pathway to prevent apoptosis," *Cell*, vol. 75, no. 2, pp. 241–251, 1993.

[14] D. J. Kane, T. A. Sarafian, R. Anton, et al., "Bcl-2 inhibition of neural death: decreased generation of reactive oxygen species," *Science*, vol. 262, no. 5137, pp. 1274–1277, 1993.

[15] J. M. Dypbukt, M. Ankarcrona, M. Burkitt, et al., "Different prooxidant levels stimulate growth, trigger apoptosis, or produce necrosis of insulin-secreting RINm5F cells. The role of intracellular polyamines," *Journal of Biological Chemistry*, vol. 269, no. 48, pp. 30553–30560, 1994.

[16] T. M. Buttke and P. A. Sandstrom, "Oxidative stress as a mediator of apoptosis," *Immunology Today*, vol. 15, no. 1, pp. 7–10, 1994.

[17] P. A. Sandstrom, M. D. Mannie, and T. M. Butke, "Inhibition of activation-induced death in T cell hybridomas by thiol antioxidants: oxidative stress as a mediator of apoptosis," *Journal of Leukocyte Biology*, vol. 55, no. 2, pp. 221–226, 1994.

[18] N. Yamauchi, H. Kuriyama, N. Watanabe, H. Neda, M. Maeda, and Y. Niitsu, "Intracellular hydroxyl radical production induced by recombinant human tumor necrosis factor and its implication in the killing of tumor cells in vitro," *Cancer Research*, vol. 49, no. 7, pp. 1671–1675, 1989.

[19] R. J. Zimmerman, A. Chan, and S. A. Leadon, "Oxidative damage in murine tumor cells treated in vitro by recombinant human tumor necrosis factor," *Cancer Research*, vol. 49, no. 7, pp. 1644–1648, 1989.

[20] D. J. Chang, G. M. Ringold, and R. A. Heller, "Cell killing and induction of manganous superoxide dismutase by tumor necrosis factor-α is mediated by lipoxygenase metabolites of arachidonic acid," *Biochemical and Biophysical Research Communications*, vol. 188, no. 2, pp. 538–546, 1992.

[21] K. Hirose, D. L. Longo, J. J. Oppenheim, and K. Matsushima, "Overexpression of mitochondrial manganese superoxide dismutase promotes the survival of tumor cells exposed to interleukin-1, tumor necrosis factor, selected anticancer drugs, and ionizing radiation," *FASEB Journal*, vol. 7, no. 2, pp. 361–368, 1993.

[22] M. Soriani, D. Pietraforte, and M. Minetti, "Antioxidant potential of anaerobic human plasma: role of serum albumin and thiols as scavengers of carbon radicals," *Archives of Biochemistry and Biophysics*, vol. 312, no. 1, pp. 180–188, 1994.

[23] M.-K. Cha and I.-H. Kim, "Glutathione-linked thiol peroxidase activity of human serum albumin: a possible antioxidant role of serum albumin in blood plasma," *Biochemical and Biophysical Research Communications*, vol. 222, no. 2, pp. 619–625, 1996.

[24] R. Hurst, Y. Bao, S. Ridley, and G. Williamson, "Phospholipid hydroperoxide cysteine peroxidase activity of human serum albumin," *Biochemical Journal*, vol. 338, part 3, pp. 723–728, 1999.

[25] D. A. Hildeman, T. Mitchell, T. K. Teague, et al., "Reactive oxygen species regulate activation-induced T cell apoptosis," *Immunity*, vol. 10, no. 6, pp. 735–744, 1999.

[26] D. A. Hildeman, T. Mitchell, B. Aronowt, S. Wojciechowski, J. Kappler, and P. Marrack, "Control of Bcl-2 expression by reactive oxygen species," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 100, no. 25, pp. 15035–15040, 2003.

[27] D. A. Hildeman, T. Mitchell, J. Kappler, and P. Marrack, "T cell apoptosis and reactive oxygen species," *Journal of Clinical Investigation*, vol. 111, no. 5, pp. 575–581, 2003.

[28] P. Marrack and J. Kappler, "Control of T cell viability," *Annual Review of Immunology*, vol. 22, pp. 765–787, 2004.

[29] M. Castedo, A. Macho, N. Zamzami, et al., "Mitochondrial perturbations define lymphocytes undergoing apoptotic depletion in vivo," *European Journal of Immunology*, vol. 25, no. 12, pp. 3277–3284, 1995.

[30] S. D. Goldstone and N. H. Hunt, "Redox regulation of the mitogen-activated protein kinase pathway during lymphocyte activation," *Biochimica et Biophysica Acta*, vol. 1355, no. 3, pp. 353–360, 1997.

[31] S. Devadas, L. Zaritskaya, S. G. Rhee, L. Oberley, and M. S. Williams, "Discrete generation of superoxide and hydrogen peroxide by T cell receptor stimulation: selective regulation of mitogen-activated protein kinase activation and Fas ligand expression," *Journal of Experimental Medicine*, vol. 195, no. 1, pp. 59–70, 2002.

[32] A. B. Lyons, "Analysing cell division in vivo and in vitro using flow cytometric measurement of CFSE dye dilution," *Journal of Immunological Methods*, vol. 243, no. 1-2, pp. 147–154, 2000.

[33] M. G. Ormerod, "The study of apoptotic cells by flow cytometry," *Leukemia*, vol. 12, no. 7, pp. 1013–1025, 1998.

[34] I. Vermes, C. Haanen, and C. Reutelingsperger, "Flow cytometry of apoptotic cell death," *Journal of Immunological Methods*, vol. 243, no. 1-2, pp. 167–190, 2000.

[35] C. A. Smith, G. T. Williams, R. Kingston, E. J. Jenkinson, and J. J. T. Owen, "Antibodies to CD3/T-cell receptor complex induce death by apoptosis in immature T cells in thymic cultures," *Nature*, vol. 337, no. 6203, pp. 181–184, 1989.

[36] A. I. Sperling and J. A. Bluestone, "The complexities of T-cell co-stimulation: CD28 and beyond," *Immunological Reviews*, vol. 153, pp. 155–182, 1996.

[37] M. L. Baroja, K. Lorre, F. Van Vaeck, and J. L. Ceuppens, "The anti-T cell monoclonal antibody 9.3 (anti-CD28) provides a helper signal and bypasses the need for accessory cells in T cell activation with immobilized anti-CD3 and mitogens," *Cellular Immunology*, vol. 120, no. 1, pp. 205–217, 1989.

[38] J. C. Rathmell and C. B. Thompson, "Pathways of apoptosis in lymphocyte development, homeostasis, and disease," *Cell*, vol. 109, no. 2, supplement 1, pp. S97–S107, 2002.

[39] J. Sprent and C. D. Surh, "Generation and maintenance of memory T cells," *Current Opinion in Immunology*, vol. 13, no. 2, pp. 248–254, 2001.

[40] S. Dohi, T. Norimura, N. Kunugita, and T. Tsuchiya, "Determination of in vivo and in vitro radiosensitivity of mouse splenic T-lymphocytes using a T-cell cloning technique," *Journal of UOEH*, vol. 11, no. 1, pp. 13–22, 1989.

[41] V. Goossens, J. Grooten, K. De Vos, and W. Fiers, "Direct evidence for tumor necrosis factor-induced mitochondrial reactive oxygen intermediates and their involvement in cytotoxicity," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 92, no. 18, pp. 8115–8119, 1995.

[42] J. Iglesias, V. E. Abernethy, Z. Wang, W. Lieberthal, J. S. Koh, and J. S. Levine, "Albumin is a major serum survival factor for renal tubular cells and macrophages through scavenging of ROS," *American Journal of Physiology*, vol. 277, no. 5, part 2, pp. F711–F722, 1999.

[43] M. E. Holt, M. E. T. Ryall, and A. K. Campbell, "Albumin inhibits human polymorphonuclear leucocyte luminol-dependent chemiluminescence: evidence for oxygen radical scavenging," *British Journal of Experimental Pathology*, vol. 65, no. 2, pp. 231–241, 1984.

[44] A. M. Cantin, B. Paquette, M. Richter, and P. Larivée, "Albumin-mediated regulation of cellular glutathione and nuclear factor kappa B activation," *American Journal of Respiratory and Critical Care Medicine*, vol. 162, no. 4, part 1, pp. 1539–1546, 2000.

[45] E. Margoliash, A. Novogrodsky, and A. Schejter, "Irreversible reaction of 3-amino-1:2:4-triazole and related inhibitors with the protein of catalase," *The Biochemical Journal*, vol. 74, pp. 339–348, 1960.

[46] P. Ou and S. P. Wolff, "Erythrocyte catalase inactivation (H_2O_2 production) by ascorbic acid and glucose in the presence of aminotriazole: role of transition metals and relevance to diabetes," *Biochemical Journal*, vol. 303, part 3, pp. 935–939, 1994.

[47] A. Takeuchi, T. Miyamoto, K. Yamaji, et al., "A human erythrocyte-derived growth-promoting factor with a wide target cell spectrum: identification as catalase," *Cancer Research*, vol. 55, no. 7, pp. 1586–1589, 1995.

[48] K. Aoshiba, Y. Nakajima, S. Yasui, J. Tamaoki, and A. Nagai, "Red blood cells inhibit apoptosis of human neutrophils," *Blood*, vol. 93, no. 11, pp. 4006–4010, 1999.

[49] U. Karrer, A. Althage, B. Odermatt, et al., "On the key role of secondary lymphoid organs in antiviral immune responses studied in alymphoplastic (aly/aly) and spleenless (Hox11(-)/-) mutant mice," *Journal of Experimental Medicine*, vol. 185, no. 12, pp. 2157–2170, 1997.

[50] B. Ludewig, B. Odermatt, S. Landmann, H. Hengartner, and R. M. Zinkernagel, "Dendritic cells induce autoimmune diabetes and maintain disease via de novo formation of local lymphoid tissue," *Journal of Experimental Medicine*, vol. 188, no. 8, pp. 1493–1501, 1998.

[51] R. M. Zinkernagel, "Localization dose and time of antigens determine immune reactivity," *Seminars in Immunology*, vol. 12, no. 3, pp. 163–171, 2000.

[52] R. M. Zinkernagel, "Immunity 2000," *Immunology Today*, vol. 21, no. 9, pp. 422–423, 2000.

[53] R. M. Zinkernagel and H. Hengartner, "Regulation of the immune response by antigen," *Science*, vol. 293, no. 5528, pp. 251–253, 2001.

[54] A. Lanzavecchia and F. Sallusto, "Dynamics of T lymphocyte responses: intermediates, effectors, and memory cells," *Science*, vol. 290, no. 5489, pp. 92–97, 2000.

[55] K. Kannan and S. K. Jain, "Oxidative stress and apoptosis," *Pathophysiology*, vol. 7, no. 3, pp. 153–163, 2000.

[56] A. Meister, "Glutathione-ascorbic acid antioxidant system in animals," *Journal of Biological Chemistry*, vol. 269, no. 13, pp. 9397–9400, 1994.

[57] F. J. T. Staal, M. T. Anderson, G. E. J. Staal, L. A. Herzenberg, C. Gitler, and L. A. Herzenberg, "Redox regulation of signal transduction: tyrosine phosphorylation and calcium influx," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 91, no. 9, pp. 3619–3622, 1994.

[58] D. E. Hutter, B. G. Till, and J. J. Greene, "Redox state changes in density-dependent regulation of proliferation," *Experimental Cell Research*, vol. 232, no. 2, pp. 435–438, 1997.

[59] J. P. Messina and D. A. Lawrence, "Cell cycle progression of glutathione-depleted human peripheral blood mononuclear cells is inhibited at S phase," *Journal of Immunology*, vol. 143, no. 6, pp. 1974–1981, 1989.

[60] M. Suthanthiran, M. E. Anderson, V. K. Sharma, and A. Meister, "Glutathione regulates activation-dependent DNA synthesis in highly purified normal human T lymphocytes stimulated via the CD2 and CD3 antigens," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 87, no. 9, pp. 3343–3347, 1990.

[61] S. Mihm, D. Galter, and W. Droge, "Modulation of transcription factor NFκB activity by intracellular glutathione levels and by variations of the extracellular cysteine supply," *FASEB Journal*, vol. 9, no. 2, pp. 246–252, 1995.

[62] M. J. Smyth, "Glutathione modulates activation-dependent proliferation of human peripheral blood lymphocyte populations without regulating their activated function," *Journal of Immunology*, vol. 146, no. 6, pp. 1921–1927, 1991.

[63] T. Peters Jr., "Serum albumin," *Advances in Protein Chemistry*, vol. 37, pp. 161–245, 1985.

[64] T. P. KING, "On the sulfhydryl group of human plasma albumin," *The Journal of Biological Chemistry*, vol. 236, p. PC5, 1961.

[65] R. M. Gatti, R. Radi, and O. Augusto, "Peroxynitrite-mediated oxidation of albumin to the protein-thiyl free radical," *FEBS Letters*, vol. 348, no. 3, pp. 287–290, 1994.

[66] R. Pirisino, P. Di Simplicio, G. Ignesti, G. Bianchi, and P. Barbera, "Sulfhydryl groups and peroxidase-like activity of albumin as scavenger of organic peroxides," *Pharmacological Research Communications*, vol. 20, no. 7, pp. 545–552, 1988.

[67] S. Z. Lei, Z.-H. Pan, S. K. Aggarwal, et al., "Effect of nitric oxide production on the redox modulatory site of the NMDA receptor-channel complex," *Neuron*, vol. 8, no. 6, pp. 1087–1099, 1992.

[68] M. Meyer, H. L. Pahl, and P. A. Baeuerle, "Regulation of the transcription factors NF-κB and AP-1 by redox changes," *Chemico-Biological Interactions*, vol. 91, no. 2-3, pp. 91–100, 1994.

[69] R. Dziarski, "Cell-bound albumin is the 70-kDa peptidoglycan-, lipopolysaccharide-, and lipoteichoic acid-binding protein on lymphocytes and macrophages," *Journal of Biological Chemistry*, vol. 269, no. 32, pp. 20431–20436, 1994.

[70] J. M. Torres, M. Geuskens, and J. Uriel, "Activated human T lymphocytes express albumin binding proteins which cross-react with alpha-fetoprotein," *European Journal of Cell Biology*, vol. 57, no. 2, pp. 222–228, 1992.

[71] A. R. Giandomenico, G. E. Cerniglia, J. E. Biaglow, C. W. Stevens, and C. J. Koch, "The importance of sodium pyruvate in assessing damage produced by hydrogen peroxide," *Free Radical Biology and Medicine*, vol. 23, no. 3, pp. 426–434, 1997.

[72] F. J. Darfler and P. A. Insel, "Clonal growth of lymphoid cells in serum-free media requires elimination of H_2O_2 toxicity," *Journal of Cellular Physiology*, vol. 115, no. 1, pp. 31–36, 1983.

[73] P. E. Purdue, S. M. Castro, V. Protopopov, and P. B. Lazarow, "Targeting of human catalase to peroxisomes is dependent upon a novel C-terminal peroxisomal targeting sequence," *Annals of the New York Academy of Sciences*, vol. 804, pp. 775–776, 1996.